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# BORDEN'S REVIEW *of* NUTRITION RESEARCH

## *A NEW VITAMIN FOR GUINEA PIGS*

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# BORDEN'S REVIEW
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# *A NEW VITAMIN FOR GUINEA PIGS*

By **RUTH WOODS**

More than ten years ago, a new dietary essential was postulated to be of importance in the nutrition of guinea pigs. Since that time the existence of this factor, its chemical nature and physiological role have been studied by several groups of investigators. The findings of these groups have been conflicting in nature, many investigators being unable to demonstrate either the existence of this factor or even evidence for its requirement by the guinea pig. For a time, it seemed likely that the new factor must be regarded as extremely hypothetical, if not actually non-existent. However, beginning a little more than a year ago, confirmation of the existence of such a factor was finally obtained by several groups of investigators other than the original workers. The new findings also confirmed the nature of the deficiency syndrome caused by a lack of this factor and, further, established the biological activity of a number of synthetic sterols and natural products in curing the deficiency. At the present time, therefore, the existence of a new dietary factor a deficiency of which has been shown to produce a definite disease entity in guinea pigs has been generally accepted. Further investigation of this factor, of its chemical identity and of its physiological role in guinea pigs is now actively underway.

## *Discovery of the New Factor:*

In 1931, Alice Bahrs (1) at Oregon State College published a report on the use of the planarian worm as an instrumental tool for demonstrating changes in the composition of mammalian tissues. This was accomplished by feeding such tissues to planarian worms and observing the various effects on these worms. In 1936, this principle was applied to studies of a nutritional nature. At that time, Bahrs and Wulzen (2) fed tissues of vitamin depleted guinea pigs and rats to planarian worms and noted the subsequent development of a characteristic disease in the worms. They observed that guinea pigs fed grain diets lacking in green feeds, but otherwise adequate—even to containing all the known vitamins—developed degenerative changes in the skeletal musculature. The condition did not develop if fresh kale or fresh alfalfa were included in the diet.

Abandoning the studies with planaria, an investigation was begun as to the nature and cause of the disorder in the guinea pigs themselves. By 1941

Wulzen and Bahrs (3), using basic milk diets in place of the grain diet had established the following facts:

**Raw Milk and Raw Cream:** Groups of young guinea pigs were fed rations of raw whole milk, pasteurized whole milk, raw skim milk and pasteurized skim milk. To these milk diets were added 10 per cent skim milk powder, adequate amounts of iron and copper, carotene, orange juice, straw and iodized salt.

Animals fed raw whole milk grew excellently and, at autopsy, showed no abnormalities of any kind. Those on heated whole milk failed to grow well and developed a peculiar stiffness of the wrist. In severe cases, the stiffness increased progressively and fatalities resulted after periods ranging from one month to a year or more. These findings, as well as further studies with skim milk, indicated the necessity for a substance present in the fat fraction of milk. The absence of the protective factor in heated milks indicated further that the factor was susceptible to destruction by heat. These assumptions were readily confirmed by the fact that raw cream or an extract of the cream given by mouth completely cured the symptom of wrist stiffness.

**Isolation of the Raw Cream Factor:** It was found that one gram of raw cream per day was sufficient to cure the disease within five days. Heating the cream in the presence of air or oxygen destroyed the factor. Cream heated in an atmosphere of nitrogen, however, remained fully active. Preliminary investigations of an active extract of cream suggested that the curative principle was methyl vinyl ketone (4), but this was later found to be inactive as well as toxic (5). In 1943, van Wagtendonk and Wulzen (5) isolated in a highly concentrated, nearly pure form, a factor from raw cream which was found to be curative in a daily dosage of 0.1 microgram within five days—ten million times as potent as the original cream.

**Assay Procedure:** Animals on the skim milk diet were found to develop the wrist stiffness syndrome in about a month. Such animals were then used to test the activity of various substances in the following way:

> The foreleg of the guinea pig on the opposite side from the experimenter was extended posteriorly close to the body wall of the animal by pressing the thumb on the "elbow" and at the same time supporting the proximal and distal parts of the leg with the fingers. The leg was held as straight as possible. The experimenter's free hand was then used to superextend the foot gently. The foot of a normal animal would bend easily until it formed a right angle with the leg. The nutritionally deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending. The angle between the foot and the leg varied from 90° in unaffected animals to 180° in very sick animals. The stiffness disappeared if active fractions of raw

cream were administered. *A unit of activity* was arbitrarily defined as a solution of an active fraction in vegetable oil (inactive carrier) which, if when administered in amounts of 1 cc. daily, cures an affected animal in five days.

**Nature of the Deficiency Syndrome:** In addition to the characteristic symptom of wrist stiffness, the dietary absence of the raw cream factor resulted in other specific deficiency manifestations. The animals became extremely weak and emaciated. Upon autopsy the muscles were found to be severely atrophied and, in most cases, were streaked with fine lines of hard calcium deposits. There were also frequently occurring calcified lumps under the skin, in the joints, between the ribs and in many organs, including the heart. The syndrome was aggravated by the feeding of cod liver oil, and although the symptoms were, in many respects, characteristic of the nutritional muscular dystrophy of vitamin E deficiency, administration of this vitamin was ineffectual.

## *Biochemical Defects Associated with Lack of Anti-Stiffness Factor:*

In an attempt to understand the role of the postulated anti-stiffness factor and the mechanisms involved in the production of the deficiency syndrome, van Wagtendonk and associates undertook a detailed investigation of the various metabolic changes associated with this condition. Following is a brief summary of their findings:

1. *Creatine excretion:* An increased excretion of creatine is characteristic of the muscular dystrophy associated with vitamin E. Since severe muscular atrophy was observed in advance cases of anti-stiffness factor deficiency, studies on creatine excretion were made (6). No significant change in creatine excretion was noted. On a combined deficiency of vitamin E and the anti-stiffness factor creatinuria and wrist stiffness developed simultaneously and independently of each other and required both factors for their correction. The creatine excretion in the combined deficiency, however, was greater than that occurring in vitamin E deficiency alone, indicating some influence of the anti-stiffness factor on creatine turnover.

2. *Phosphorus metabolism:* Because of the occurrence of abnormal deposits of calcium phosphate in the deficiency condition, the possibility of a derangement in phosphorus metabolism was suggested and investigated. Such a derangement was found as evidenced by changes in the distribution of various phosphate fractions in the liver and kidneys (7). Inorganic phosphorus increased markedly in both organs. Other phosphorus fractions, representing a large percentage of the body's ADP and ATP* dropped sharply. Nucleotide phosphorus

*Adenosinediphosphate and adenosinetriphosphate, respectively — compounds involved in energy transport and muscle metabolism; *cf. Borden's Review of Nutrition Research*, May, 1948, "Modern Concepts of Food Utilization."

increased. The abnormal distribution of acid-soluble phosphorus was prevented by the anti-stiffness factor which also caused a return to normal distribution if given after the derangement had occurred. These changes suggested that significant alterations in ADP and ATP occur in the wrist-stiffness syndrome, possibly involving the whole metabolism of purines.
Further studies showed also a decline in serum phosphatase in deficient guinea pigs—a condition common in scurvy. However, vitamin C did not prevent this decline, although prolonged administration of the anti-stiffness factor did (8).

3. *Blood Changes:* Both calcium and inorganic phosphorus were found to be increased in the blood during deficiency (9), a condition favoring the formation of colloidal calcium phosphate and its subsequent deposition in muscle tissues. Anti-stiffness factor causes a return to normal levels of blood calcium and phosphorus. With time, or with prolonged administration of high dosages of the anti-stiffness factor, the deposits are slowly removed from the tissues as well.

4. *Muscle Changes:* A deranged distribution of the acid-soluble phosphorus, comparable to that observed in liver and kidney, was observed in muscle as well (10). Inorganic phosphorus increased, while creatine phosphate and ATP declined.

5. *Plasma Protein Changes:* Because blood calcium is involved in an intimate balance with blood proteins—presumably as free calcium ions and as calcium bound to the albumin fraction (11)—investigation of changes in plasma protein distribution during the deficiency condition was made. As expected such changes had occurred. Plasma globulin was increased, but serum albumin was markedly lower. With the loss of albumin, there was also a loss of the albumin-bound calcium. In view of the increased total calcium observed during this deficiency this meant that the free calcium in the blood had increased. Thus, higher concentrations of free calcium and of inorganic phosphate favor the formation and deposition of colloidal calcium phosphate.

**Interpretation of Biochemical Changes:** van Wagtendonk and coworkers have interpreted these findings as part of a pattern of events occurring during a deficiency of the anti-stiffness factor. The deranged phosphorus metabolism in the liver and kidney lead to a disturbance of normal liver function. This, in turn, interferes with the normal synthesis of protein. With the decrease in serum albumin (containing bound calcium), the ratio of free calcium in the blood increases. Similarly, in muscles, creatine phosphate and ATP (containing bound phosphorus) decrease and inorganic phosphorus increases. As a result of the higher concentrations of free calcium and inorganic phosphorus, colloidal calcium phosphate is formed which may precipitate whenever conditions are favorable. The role of the anti-stiffness factor in this sequence of events remains obscure, the possibility being that it might function—like many of the vitamins—as an enzyme or coenzyme in one or more of these reactions.

## *Negative Findings:*

At about this time investigation of the wrist stiffness syndrome in guinea pigs was investigated by two other groups, Homberger and Reed (13) and Kon and associates (14). Both of these groups failed to demonstrate wrist stiffness in guinea pigs on a skim milk diet. In addition, they reported poor condition of the animals and very high mortality on these incomplete diets. Other investigators also found these diets to be extremely poor in maintaining the animals and were led, accordingly, to develop other types of basic diets for the production of the deficiency.

## *Confirmative Findings:*

In 1947, Oleson and coworkers (15) published studies which for the first time confirmed the findings of Wulzen and Bahrs and of Wulzen, van Wagtendonk and associates. They found that guinea pigs on specially devised deficient diets developed the wrist stiffness syndrome described by the Oregon workers. They observed that dried penicillin mold pad was curative and that the activity was concentrated in the sterol fraction. Accordingly, they tested the effects of some 59 pure steroids of which more than 24 were found to be effective in preventing or curing the condition. Most active of these compounds were the sterol ergostanyl and several of its esters, such as ergostanyl acetate. These compounds gave responses in doses as low as 1 to 5 micrograms, comparable to results obtained with similar amounts of van Wagtendonk's unidentified crystalline anti-stiffness factor (16). Oleson *et al* (15) further confirmed the anti-stiffness properties of an extract from cane juice which had earlier been reported by van Wagtendonk *et al* (16) to be an even more potent source of the curative factor than raw cream. In contrast to the findings of van Wagtendonk and Wulzen, however, Oleson's group were unable to confirm the extreme potency—i.e. 0.002 mcgm. dosages —of the cane juice factor.

**The Sugar Cane Juice Factor:** The occurrence of an anti-stiffness factor in sugar cane juice was discovered by tracing the origin of the factor in raw cream to the molasses content of the cow's feed. The amount of molasses in the feed was found to have a direct influence on the amount of anti-stiffness factor found in the cream. Both molasses and sugar cane juice were found, upon investigation, to be good sources of the unidentified factor.

Although the Oregon workers (16) originally reported that as little as 0.002 mcgm. of a crystalline material extracted from cane juice was curative, they later stated (17) that the activity of the crystalline factor was due to

traces of an impurity which would make the active material even more potent —in other words, active at the incredibly small calculated dosage of 0.00002 mcgm. (18). With the inability of Oleson *et al* to confirm even the 0.002 mcgm. dosage potency, this later figure of an almost invisible quantity, would seem to be even more questionable. A number of possible discrepancies in the findings have been pointed out in a recent survey of this subject (18) which may have a bearing on the various findings reported. One of the major factors causing difficulty and confusion in this field, as agreed upon by all investigators, is the quantitative inadequacy of the present method of assay which is still too greatly a matter of subjective interpretation (15, 19).

**Other Studies:** The following year, further confirmation of the wrist stiffness syndrome appeared in the work of Petering *et al* (20). These investigators were unable to maintain animals successfully on the skim milk diet of the Oregon workers and devised another type of diet which succeeded in producing the desired deficiency condition (casein, corn starch, brewer's yeast, salts, cottonseed oil with fat-soluble vitamins, ascorbic acid). They succeeded in producing the disease on this diet and confirmed the curative properties of ergostanyl acetate (Oleson *et al*). They found, however, that about 100 mcgm. of this material were required as compared to Oleson's 1 to 5 mcgm. and van Wagtendonk's 0.002 mcgm. of unidentified crystalline material from sugar cane. Petering *et al* also found alpha-ergostanyl acetate even more active, although in dosages of the same order (100 mcgm.). They reported further that anti-stiffness activity is present in fresh cabbage and oatmeal (used in their basal diet). These workers, unlike the Oregon group, found no evidence of calcium phosphate deposition in their animals although the development of wrist stiffness was very severe in many cases.

Further interesting contributions to this problem have been made by Smith and associates (21) at Cornell University. These investigators were able to produce both the wrist stiffness and calcification syndromes in guinea pigs on various types of deficient diets. However, they failed to find any evidence for the curative properties of sugar cane juice, penicillin mold pad or ergostanyl esters. As a result of their studies and in view of the findings of Petering *et al* (20) they conclude further that the manifestations of wrist stiffness and abnormal calcium salt deposition are two separate syndromes which occur independently of each other and for different reasons.

**Possible Influences of Vitamin D, Calcium and Phosphorus Ratios in the Diets:** Detailed examination of the diets used by various investigators reveals significant variations in the levels of calcium, phosphorus and vitamin D supplied. The possibility exists that an imbalance of these factors, rather

than an actual nutritional deficiency, might be responsible for the wrist stiffness and calcification syndromes. In this connection it may be pointed out that one of the diets used by van Wagtendonk *et al* contained 10% of irradiated yeast which furnishes an unusually high, possibly toxic, level of vitamin D. It is not known, however, whether this level of vitamin D will actually cause toxic calcification in the guinea pig.

As Petering and associates (20) have pointed out, also, the calcium and phosphorus contents of the diets must be considered. They cite the findings of Hogan and Regan (22) who have reported the deposition of calcium phosphate on basal diets similar to their own and having a content of phosphorus similar to that of the skim milk diets of Wulzen and van Wagtendonk. They conclude that wrist stiffness can occur on diets which are either high or low in phosphorus (their diets calculated to have 0.58% P comparing with the lower phosphorus diet of 0.5% of Hogan and Regan), but that calcium phosphate deposition occurs readily only on a high phosphorus diet (the skim milk diet of Wulzen and van Wagtendonk having 1.0% P, comparable to the high phosphorus diet of 0.9% of Hogan and Regan). The occurrence of calcification on their original diets has been more recently confirmed by Harris and Wulzen (23) who reported in detail on the pathological changes caused in guinea pigs on diets deficient in the wrist stiffness factor.

### *Implications of These Studies:*

Although the observations of various investigators have often been at variance with one another thereby presenting a generally confused picture of the status of the new deficiency syndrome, several positive conclusions may be drawn at the present time: (1) Certain types of deficient diets result in the abnormal phenomenon of wrist stiffness in guinea pigs; (2) Such diets may also lead to abnormal calcification in various tissues of the body, although calcium-phosphorus imbalances rather than a deficiency of a specific nutrient may be involved; (3) With one exception, most research groups have found either synthetic sterols or naturally occurring products (raw cream, sugar cane juice or bagasse, penicillin mold pad, oatmeal, cabbage) to be a source of biologically active material capable of preventing or curing the wrist stiffness syndrome induced by dietary deficiency. Further studies to determine the nature and identity of the anti-stiffness factor are now underway. In addition, methods for perfecting a reliable, quantitative biological assay are being sought. These researches must be completed before the confusion in this promising field of investigation can be cleared away satisfactorily.

Other questions which naturally present themselves concern the possible need for the guinea pig anti-stiffness factor by other animal species or by man. Inasmuch as much of the existing evidence with respect to guinea pigs is still largely in a questionable stage, such questions can scarcely be considered on any rational basis at the present time. Even more remote are the possible dietary implications for man—a) the need for this factor has not even remotely been indicated and b) most of the sources of the anti-stiffness factor are raw, inedible products. Further, with respect to a product such as raw cream, only certified raw cream low in pathogenic organisms would be suitable for practical purposes. The use of non-pasteurized cream from general dairy sources would be attended with such serious health hazards as to more than offset any possible therapeutic value.

With respect to the possible role of this factor in human nutrition, it is intriguing to speculate on the possible relation between the wrist stiffness syndrome in guinea pigs and arthritis in human subjects. It will be interesting to determine the relationship, if any, between the sterols possessing anti-stiffness activity for the guinea pig and the sterols (such as 17-hydroxy-11-dehydrocorticosterone, popularly known as "compound E" or cortisone) which have been found to relieve the stiffness and other symptoms of arthritis. The existence of such relationships would have several important consequences; the guinea pig could serve as a useful tool for evaluating the anti-arthritic activity of cortisone, related compounds or potential new therapeutic agents; further, the possibility of materials possessing anti-stiffness activity for the guinea pig serving as substitutes for cortisone or for the equally scarce starting materials in its synthesis is an irresistable thought for future investigation.

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